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Application of multiple-locus variable-number tandem-repeat analysis to determine clonal spread of toxin A-negative *Clostridium difficile* in a general hospital in Buenos Aires, Argentina

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Abstract

Isolates from patients with *Clostridium difficile* infection (CDI) usually produce both toxin A (TcdA) and toxin B (TcdB), but an increasing number of reports from Europe and Asia mention infections with TcdA-negative, TcdB-positive (A−/B+) strains, usually characterized as PCR ribotype 017 (type 017). Incidence rates of CDI per 10 000 admissions in a 200-bed Argentinean general hospital were 37, 84, 67, 43, 48 and 42 for the years 2000 to 2005, respectively. The annual percentages of type 017 CDI were 7.7%, 64.6%, 91.4%, 92.0%, 75.0% and 86.4%, respectively. Comparison of 112 017-CDI patients with 41 non-017-CDI patients revealed that 017-CDI patients were more often male (68.8% vs. 46.3%; odds ratio 2.55, 95% confidence interval 1.23–5.50). All type 017 strains tested belonged to toxinotype VIII and had a 1.8-kb deletion in *tcd*A. In addition, 90% of tested type 017 isolates had high-level resistance to clindamycin and erythromycin, determined by the presence of the *erm*B gene. Multiple-locus variable-number tandem-repeat analysis (MLVA) was applied to 56 Argentinean isolates and 15 isolates from seven other countries. Country-specific clonal complexes were found in each country. Among 56 Argentinean isolates, four clonal complexes were recognized, accounting for 61% of all isolates. These clonal complexes did not show correlation over time, but seemed to be restricted to specific wards, mainly internal medicine and pulmonology wards. A total of 56% of recurrent infections were caused by a different isolate, despite identification of an identical PCR-ribotype. We conclude that *C. difficile* type 017 gradually replaced other circulating PCR ribotypes and that MLVA provides detailed insight into nosocomial spread.

Keywords: Clonal spread, *Clostridium difficile*, MLVA, PCR ribotype 017, recurrences, toxin A negative, toxinotype VIII
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Introduction

The spectrum of *Clostridium difficile*-associated infection (CDI) ranges from asymptomatic carriage to fulminant, relapsing and potentially fatal colitis [1]. Pathogenic *C. difficile* strains release the toxins A (TcdA) and B (TcdB) that ultimately mediate diarrhoea and colitis. The exact role of a third toxin (binary toxin) is still unknown [2]. The initial consensus that TcdA and TcdB act synergistically and that the action of TcdB occurs via the tissue damage caused by TcdA resulted in the view that TcdA was the most important factor for causing diarrhoeal disease [3]. However, since the discovery of TcdA-negative, TcdB-positive (A−/B+) *C. difficile* strains [4,5], at least four outbreaks [6–9] caused by these strains have been described and an increasing number of reports have mentioned clinical disease associated with these variants [10–13]. The percentage of A−/B+ strains among patients with CDI has been described as approximately 3% in the UK and France [14,15]. Recently, an increase in A−/B+ strains has also been reported from Korea [16].

The most commonly found A−/B+ strain is PCR-ribotype 017, toxinotype VIII, serogoup F, and restriction enzyme analysis (REA) group CF [11]. This strain contains a 1.8-kb dele-

tion in *tcd*A and a mutation at amino acid position 47, resulting in a stop codon and a truncated TcdA protein. Patients infected with this strain show similar clinical presentation and recurrence rates as patients infected with A+B+ strains [6,8]. Further subtyping can be performed by amplified fragment length polymorphism (AFLP), REA and multiple-locus variable-number tandem-repeat analysis (MLVA) [17–20]. MLVA has also been applied as a highly discriminatory fingerprinting system to analyse clusters of *C. difficile* infection cases caused by epidemic ribotype 027 isolates [18,21].

This report describes a nearly total clonal replacement of the heterogeneous population of *C. difficile* by A−/B+ PCR ribotype 017 (type 017) in an Argentinean hospital and compares the clinical and demographic characteristics of patients with CDI caused by type 017 (017-CDI) with those of non-017-CDI patients. In addition, MLVA was applied to study the spread of type 017 in more detail.

Materials and Methods

Study design

This study was performed from May 2000 to December 2005 in a 200-bed general hospital in Buenos Aires. During this period, faecal samples from patients with clinically suspected CDI were both cultured on selective media, after alcohol pre-treatment, for the presence of *C. difficile* [22] and tested for the presence of *C. difficile* toxins. Faecal samples were tested during the first 2 years with an enzyme immune assay (Cytoclone A/B; Meridian Diagnostics Inc., Cincinnati, OH, USA) and thereafter with a combined assay for the detection of toxin A and cell wall antigen (Triage; Biosite Diagnostics, Inc., San Diego, CA, USA). All isolates were sent to the Anaerobe Reference Laboratory in Cardiff, Wales (n = 40) and to Leiden University Medical Centre (LUMC, n = 131) for further identification.

Demographic and clinical data

Institutional board approval was obtained for the collection of demographic patient data (age, gender), data on the wards in which patients were diagnosed with CDI, and data on the HIV status of patients and recurrence rates of CDI. A CDI case was defined as that of a patient with a positive laboratory assay for *C. difficile* TcdA and/or TcdB in stools or a toxin-producing *C. difficile* organism detected in stool via culture [23]. A recurrent CDI case was defined as an episode that occurred within 8 weeks after the onset of the previous episode [23]. Demographic and clinical data for patients with type 017 were compared with equivalent data for patients with other types. A Yates-corrected chi-square test was used for the analysis of proportions. If a cell value was < 5 in the two-by-two table, Fisher's exact test was used. Crude relative risks were estimated as odds ratios (ORs) and presented with a 95% confidence interval (95% CI). All analyses were performed using spss for Windows, Version 13.0 (SPSS, Inc., Chicago, IL, USA).

Reference strains

Fifteen known type 017 isolates from seven different countries were included and used as reference strains. These strains have been characterized by MLVA and AFLP, as previously described [19,20].

Strain characterization

All isolates were genetically identified as *C. difficile* using an in-house PCR for the presence of the *glu*D gene, encoding glutamate dehydrogenase (GDH), specific for *C. difficile* [24]. All *C. difficile* strains were further investigated by PCR ribotyping as described previously [25]. The presence of the toxin genes *tcd*A and *tcd*B was investigated with standardized techniques [26,27]. Toxinotyping was performed as described by Rupnik *et al.* [28]. A random selection of strains, distributed over the study period, was tested for deletions in *tcd*C by PCR, using primers designed in-house [24], and for the presence of binary toxin genes, as described previously [29]. *C. difficile* strains were also investigated for the presence of the *erm*B gene, coding for resistance to macrolides and lincosamides.

Multiple-locus variable-number tandem-repeat analysis (MLVA)

A random selection of type 017 strains was tested by MLVA using the seven markers, CdA6, CdB7, CdC6, CdG8, CdE7, CdF3 and CdH9, as previously described, with one alteration: a new reverse primer was developed for marker CdG8: 5′ ACCAAAAATTTCTAACCCAAC 3′ [20]. The genetic relationships among the genotypes were determined by clustering them according to MLVA type using the number of differing loci and the summed absolute distance as coefficients for calculating the minimum spanning tree (MST), as described by Marsh *et al.* [18], using the bionumerics software (Version 4.6; Applied Maths NV, Sint-Martens-Latem, Belgium). Briefly, the summed absolute distance between two MLVA-typed isolates is the summed tandem-repeat difference (STRD) at all seven variable-number tandem-repeat (VNTR) loci. Isolates with an STRD ≤ 10 were defined as genetically related, irrespective of the number of differing loci. Clonal complexes were defined by an STRD ≤ 2, provided that isolates were single locus variants (SLVs) or double locus variants (DLVs) of one another [18].

Results

Incidence and strain characteristics

During the study period, 171 strains isolated from 153 patients were investigated; 131 were examined at LUMC and 40 were typed by author JSB in Cardiff, UK. Table 1 shows annual rates of admissions, CDI incidence, toxinogenic cultured isolates and type 017 isolates typed by MLVA. In total, 127 (74.3%) isolates were identified as type 017, 27 (15.8%) as type 001, nine (5.3%) as type 012 and six (3.5%) as type 014. The remaining two strains were types 031 and 039.

All 131 isolates sent to Leiden were analysed in more detail. Of these, 119 were identified as type 017, seven as type 001, four as type 014 and one as type 039. The *erm*B gene was present in 89.9% of type 017 strains, 85.8% of type 001 strains, 50% of type 014 strains and in the only type 039 strain. The percentage of *erm*B-positive strains showed an upward trend for each year, from 67.9% in 2001 to 100% in 2005. All type 017 strains had the 1.8-kb deletion in *tcd*A and contained *tcd*B. In total, nine type 017 strains were investigated by toxinotyping (seven in 2001, one in 2002 and one in 2003); all of these belonged to toxinotype VIII. In addition, none of these tested type 017 strains harboured binary toxin genes or showed deletions in *tcdC*.

Epidemiological and demographic data

Of 153 CDI patients, 112 had 017-CDI and 41 had non-017-CDI. Patients with 017-CDI were significantly more often male (68.8% vs. 46.3%; OR 2.55, 95% CI 1.23–5.30). No significant differences were found with regard to age, the ward on which CDI was acquired, HIV status or recurrence rate (9% and 12% for patients with 017-CDI and non-017-CDI, respectively).

TABLE 1. Annual number of admissions, *Clostridium difficile* infection (CDI) incidence, number of cultured toxinogenic isolates and number of type 017 isolates typed by multiple-locus variable-number tandem-repeat analysis (MLVA)

Year	Number of admissions	CDI incidence per 10 000 admissions	Number of toxinogenic culture-positive isolates		Type 017 isolates typed by MLVA (%)
			Total	Type 017 (%)	
2000	3204*	37	13*	1 (7.7)	1 (100)
2001	4500	84	48	31 (64.6)	18 (58)
2002	4759	67	35	32 (91.4)	9 (28)
2003	5347	43	25	23 (92.0)	10 (43)
2004	4978	48	28	21 (75.0)	10 (47)
2005	5265	41	22	19 (86.4)	8 (42)

*Data from May 2000.

Thirteen of the 153 patients (8.5%) developed one or more recurrences. In all patients with an initial type 017 infection (n = 8), this type was also identified in the recurrent episode(s). The other five patients had an initial infection with type 001. Among these, two had a recurrence with type 001, one with type 012 and two with type 017. The minimum time interval between the first episode and recurrence with an identical type was 15 days and the average interval was 26 days. The minimum interval between first episode and recurrence with a different type was 11 days and the average interval was 21 days (not significant).

MLVA was performed on a total of 71 isolates, including 56 Argentinean type 017 isolates and 15 type 017 isolates from seven other countries. The Argentinean isolates were derived from 45 patients and equally distributed over 2000–2005. The VNTR loci CdC6, CdG8 and CdE7 were the most variable, whereas the loci CdF3 and CdH9 were constant and common to all 017 strains (with the exception of one isolate for CdH9). In total, the 71 type 017 isolates could be subtyped into 57 unique MLVA types.

Figure 1 shows the MST of all 71 typed isolates. Each circle represents a unique MLVA type containing one or more isolates. The dark grey circles represent one large related cluster (dark grey cluster), defined by an STRD $\leq$ 10, containing 47 isolates. Within the dark grey cluster, five clonal complexes (CC-A to CC-E) were identified, defined by an STRD $\leq$ 2. Of the 56 Argentinean isolates, 42 (75%) belonged to the dark grey cluster. By contrast, five of the 15 (33%) reference strains belonged to this cluster. A total of 34 Argentinean isolates (61%) were located within clonal complexes A–D, as was one reference strain (7%). Two Argentinean clusters that were not related to the dark grey cluster (light grey circles marked I and CC-1) were SLVs of this cluster (STRDs of 16 and 25, respectively) and varied in the same locus CdG8.

Among the reference strains, three clusters were identified (light grey circles marked II and CC-2, and dark grey circles marked CC-E), all of which were country-specific. Of the reference strains, 10 (67%) had a genetic distance of one or multiple DLVs from the dark grey cluster, compared with five (9%) of the Argentinean isolates.

To further elucidate the relatedness between the 56 Argentinean type 017 isolates, we considered the ward on which CDI was diagnosed and the year of isolation in the MST (Fig. 2). Clonal complexes were found among 20 (67%) of 30 isolates from the internal medicine ward, nine (82%) of 11 isolates from the pulmonology ward, four (50%) of eight isolates from the intensive care unit, two (50%) of four isolates from the surgical ward and one (33%) of three isolates from other departments.

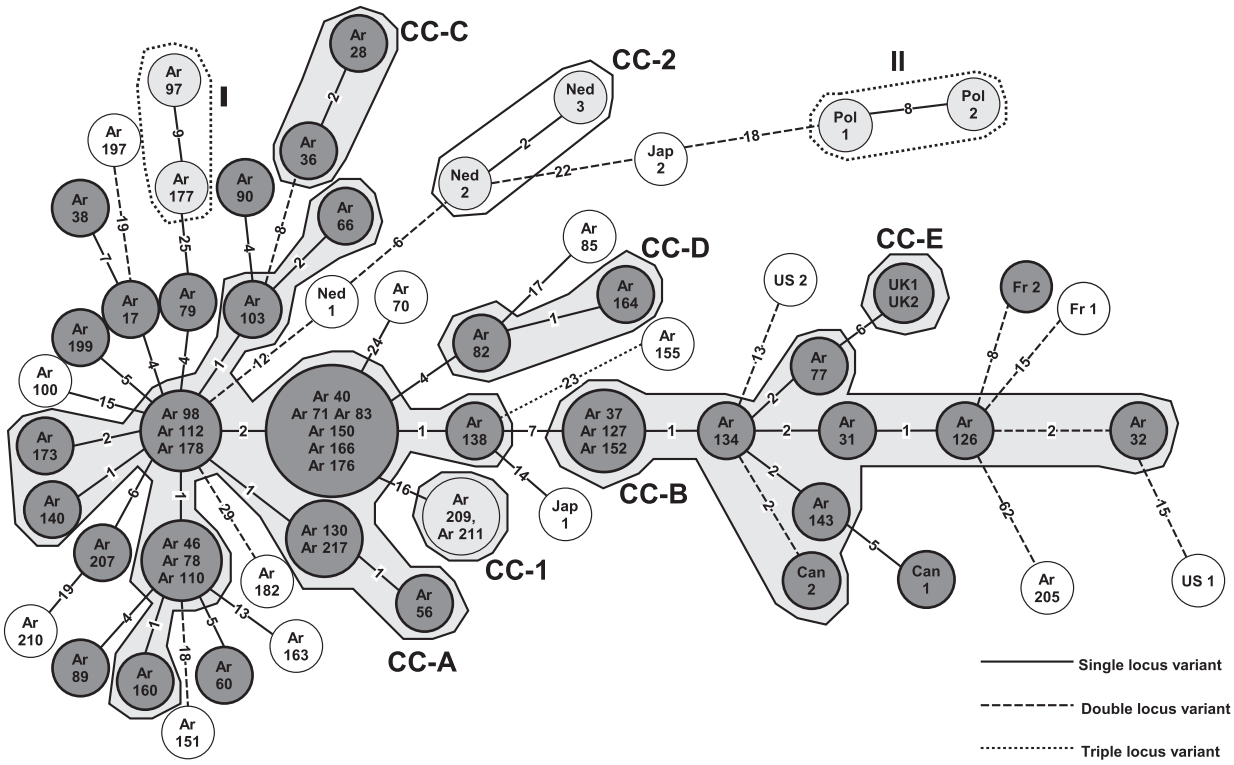

FIG. 1. Minimum spanning tree analysis of 71 *Clostridium difficile* isolates typed by multiple-locus variable-number tandem-repeat analysis (MLVA), including 56 Argentinean isolates (Ar) and 15 isolates from other countries. Isolates are labelled by country plus a number. Unique isolates or isolates with completely identical MLVA types are shown within a dark grey, light grey or white circle. The numbers between the circles represent the summed tandem repeat differences (STRDs) between MLVA types. All isolates in dark grey circles represent a large genetically related cluster, defined by an STRD ≤ 10. Within the light grey cluster, five boxed clonal complexes (CC-A to CC-E) are defined by an STRD ≤ 2. Isolates in light grey circles are genetically related clusters (clusters I and II in dashed boxes) or clonal complexes (CC-1 and CC-2) that do not belong to the large dark grey cluster. The white circles represent isolates that are not related to light or dark grey isolates. Ned, the Netherlands; Pol, Poland; Jap, Japan; US, USA; Fr, France; Can, Canada.

The 56 Argentinean isolates were derived from 45 patients. Seven of these patients had one and one patient had two recurrences of type 017. Table 2 presents the time interval between episodes, number of differing loci and STRD. Using the same definitions for clonality, four of nine recurrences (44%) were caused by an identical isolate. Of these, three differed only on locus CdC6 and one only on locus CdG8. By contrast, five of nine recurrences (56%) were caused by different isolates. Of these, one was an SLV (STRD of 4) and differed only on locus CdE7. The other four recurrences were DLVs (STRDs of 19, 24, 29 and 30, respectively), of which three differed on loci CdE7 and CdG8 and one differed on loci CdC6 and CdG8.

Discussion

During a period of 5 years, a clonal expansion of *C. difficile* PCR ribotype 017 was noticed, in which type 017 increasingly replaced other types. In 2001, the incidence doubled, from 37 to 84 per 10 000 admissions, after which it receded to the previous endemic rate, approximating 40 per 10 000 admissions. The contribution of 017-CDI to the overall incidence, however, remained very high. Clonal spread of type 017 has only been described in outbreak situations [6–8,30] and this is the first report of a persisting high incidence of type 017 CDI in one hospital. The finding that significantly more male patients were affected by type 017 is of interest, but no firm conclusions can be drawn as no information was available on the male : female ratios of patients admitted to the hospital and to specific wards. The recurrence rate for type 017 (9%) was comparable with recurrence rates for other PCR ribotypes (12%) and with the recurrence rates of this strain described in outbreak situations [6,8].

Application of MLVA resulted in the recognition of 57 unique MLVA types among 71 type 017 isolates, encompassing 56 Argentinean isolates and 15 isolates from other countries. When the isolates (n = 4) from a recurrent infection

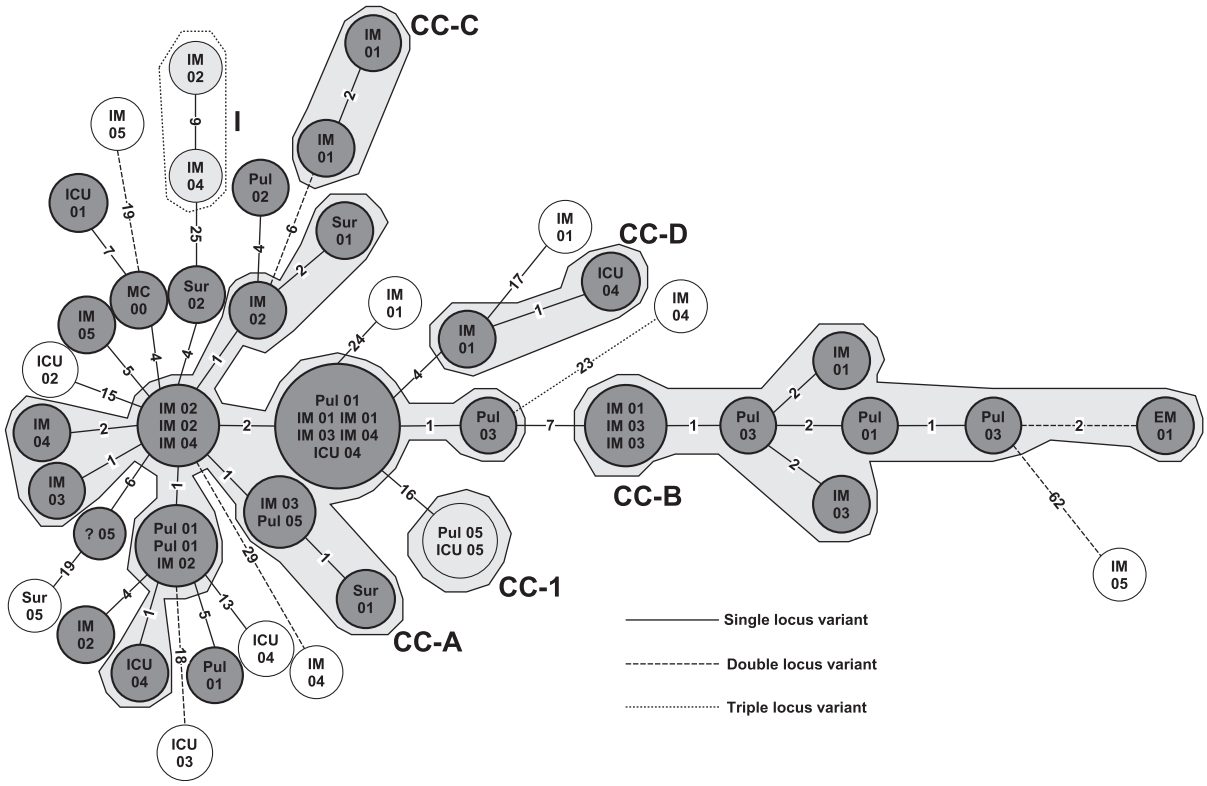

FIG. 2. Minimum spanning tree of 56 Argentinean isolates, derived from Fig. 1, by ward and year. Isolates are labelled by department and the last two digits of the year of isolation. Unique isolates or isolates with completely identical multiple-locus variable-number tandem-repeat analysis (MLVA) types are shown within dark grey, light grey or white circles. The numbers between the circles represent the summed tandem repeat differences (STRDs) between MLVA types. All isolates in dark grey circles represent a large genetically related cluster, defined by an STRD ≤ 10. Within the dark grey cluster, four boxed clonal complexes (CC-A to CC-D) are defined by an STRD ≤ 2. Isolates in light grey circles are a genetically related cluster (cluster I in a dashed box) and a clonal complex (CC-1) that do not belong to the large dark grey cluster. The white circles represent isolates that are not related to light or dark grey isolates. IM, internal medicine; Pul, pulmonology; ICU, intensive care unit; Sur, surgery; MC, medium care; EM, emergency medicine.

with an identical strain by MLVA were excluded from the analysis, the structure of the spanning tree did not significantly change (data not shown). Argentinean isolates could clearly be discriminated from isolates of other countries. A total of 75% of the Argentinean isolates were genetically related. Of the Argentinean isolates that were not related, 16% were SLVs and only 9% showed a greater genetic distance. By contrast, 33% of the isolates from other countries were found in the related Argentinean cluster, whereas 67% were not related, all of which were double or triple locus variants. In addition, all clonal complexes were country-specific (with the exception of one Canadian isolate in CC-B). Among the Argentinean isolates, clonal complexes did not show a correlation over time. Instead, these complexes seemed to be restricted to specific wards, mainly those for internal medicine and pulmonology, where 67% and 82% of isolates, respectively, belonged to a clonal complex. This observation suggests the continuous presence of clones on these wards, providing a source for new infections at various time intervals.

MLVA is a highly discriminatory typing method for *C. difficile* [18,20]. Killgore *et al.* [31] recently examined the discriminatory power of seven DNA fingerprinting techniques when applied to 42 *C. difficile* strains collected in four countries. Only REA and MLVA had sufficient power to distinguish strains from different outbreaks, and to discriminate between North American and European type 027 isolates. Recently, MLVA was compared with pulsed field gel electrophoresis (PFGE) to determine its ability to identify *C. difficile* clusters among 91 type 027 isolates from nine hospitals in the UK. MLVA was far superior to PFGE for analysing clusters of CDI both within and between institutions [21].

The finding that a major proportion of recurrences is caused by re-infection rather than relapse has been reported before [32,33]. The difference here is that we found a majority (56%) of recurrences that were caused by a different

TABLE 2. Application of multiple-locus variable-number tandem-repeat analysis MLVA to isolates from eight patients with type 017 *Clostridium difficile* infection with recurrent infections with type 017

Patient	Episode	Sample number	Time interval, days	Differing locus or loci	STRD	Same or different isolate*
1	First	Ar 40	33	CdC6	1	Same
	Second	Ar 46				
2	First	Ar 56	38	CdC6	1	Same
	Second	Ar 66				
3	First	Ar 110	24	CdC6	1	Same
	Second	Ar 112				
4	First	Ar 173	18	CdG8	2	Same
	Second	Ar 178				
	Third	Ar 178	21	CdE7,CdG8	29	Different
		Ar 182				
5	First	Ar 71	24	CdE7	4	Different
	Second	Ar 82				
6	First	Ar 98	23	CdE7, CdG8	30	Different
	Second	Ar 97				
7	First	Ar 160	24	CdE7, CdG8	24	Different
	Second	Ar 163				
8	First	Ar 209	28	CdE6, CdG8	19	Different
	Second	Ar 217				

STRD, summed tandem-repeat difference.
*Isolates were defined as identical (clonal complex) if the STRD between first infection and recurrence was ≤ 2 in combination with two or less differing loci. The annotation of the strains corresponds with that in Fig. 1.

strain, despite identification of the identical PCR-ribotype in initial infection and recurrence. This may be of clinical relevance because the treatment approach towards a re-infection with a different strain differs from the approach taken for recurrent infections with the same strain. However, it remains difficult to distinguish between a re-infection with a different strain and a relapse with the same endogenous strain. The first could represent a relapse with a different previously unrecognized endogenous strain; the second could represent a re-infection from the environment with the same circulating strain. Van den Berg *et al.* [34] showed that different PCR ribotypes could be found simultaneously in stool samples of two of 23 patients with CDI. By contrast, O'Neill *et al.* found that all 10 cultured colonies of each of 10 patients with a first episode of CDI contained the same REA type [32].

Important questions that arise from this report concern how clinical replacement strain has established itself and persisted. One explanation may reflect increased selection pressure induced by antibiotic usage. Unfortunately, we were not able to correlate CDI incidence rates with antibiotic usages in daily defined dosages in the different departments. Another explanation may involve altered virulence properties of type 017 isolates. All tested type 017 strains belonged to toxinotype VIII, did not harbour binary toxin genes and contained an intact *tcd*C, and 90% of type 017 isolates tested harboured the *erm*B gene, coding for resistance to macrolides and lincosamides. Together, these observations are not indicative of altered virulence characteristics, as observed for PCR ribotype 027 strains.

It is very likely that, despite infection control measures, spores of type 017 persisted in the hospital and were able to cause continued transmission. This occurred especially in the internal medicine and pulmonology wards. This persistence may have been caused by contamination of the environment or by ongoing spread through asymptomatic carriers, which has recently been suggested as an important risk factor for nosocomial transmission [35]. Our finding that 55% of recurrences were infections with a different 017 MLVA type supports the hypothesis that the environment and other patients or healthcare workers contributed to the mode of transmission significantly.

We conclude that *C. difficile* type 017 gradually replaced other circulating PCR ribotypes and was associated with a similar spectrum of clinical disease as that known for A+/B+ strains. Using MLVA, a more detailed insight into nosocomial transmission of 017 isolates was obtained. Further research is needed to understand the clonal spread and longterm epidemiology of type 017.

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Transparency Declaration

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References

1. Bartlett JG. Clinical practice. Antibiotic-associated diarrhoea. *N Engl J Med* 2002; 346: 334–339.
2. Geric B, Carman RJ, Rupnik M *et al.* Binary toxin-producing, large clostridial toxin-negative *Clostridium difficile* strains are enterotoxic but do not cause disease in hamsters. *J Infect Dis* 2006; 193: 1143–1150.
3. Lyerly DM, Saum KE, MacDonald DK, Wilkins TD. Effects of *Clostridium difficile* toxins given intragastrically to animals. *Infect Immun* 1985; 47: 349–352.
4. Lyerly DM, Barroso LA, Wilkins TD, Depitre C, Corthier G. Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile*. *Infect Immun* 1992; 60: 4633–4639.

5. Moncrief JS, Zheng L, Neville LM, Lyerly DM. Genetic characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates by PCR. *J Clin Microbiol* 2000; 38: 3072–3075.
6. Alfa MJ, Kabani A, Lyerly D *et al.* Characterization of a toxin A-negative, toxin B-positive strain of *Clostridium difficile* responsible for a nosocomial outbreak of *Clostridium difficile*-associated diarrhoea. *J Clin Microbiol* 2000; 38: 2706–2714.
7. Drudy D, Harnedy N, Fanning S, O'Mahony R, Kyne L. Isolation and characterization of toxin A-negative, toxin B-positive *Clostridium difficile* in Dublin, Ireland. *Clin Microbiol Infect* 2007; 13: 298–304.
8. Kuijper EJ, de Weerdt J, Kato H *et al.* Nosocomial outbreak of *Clostridium difficile*-associated diarrhoea due to a clindamycin-resistant enterotoxin A-negative strain. *Eur J Clin Microbiol Infect Dis* 2001; 20: 528–534.
9. Sato H, Kato H, Koiwai K, Sakai C. A nosocomial outbreak of diarrhoea caused by toxin A-negative, toxin B-positive *Clostridium difficile* in a cancer centre hospital. *Kansenshogaku Zasshi* 2004; 78: 312–319.
10. Kikkawa H, Hitomi S, Watanabe M. Prevalence of toxin A-non-producing/toxin-B-producing *Clostridium difficile* in the Tsukuba-Tsuchiura district, Japan. *J Infect Chemother* 2007; 13: 35–38.
11. Drudy D, Fanning S, Kyne L. Toxin A-negative, toxin B-positive *Clostridium difficile*. *Int J Infect Dis* 2007; 11: 5–10.
12. Pituch H, van den BN, van Leeuwen W *et al.* Clonal dissemination of a toxin-A-negative/toxin-B-positive *Clostridium difficile* strain from patients with antibiotic-associated diarrhoea in Poland. *Clin Microbiol Infect* 2001; 7: 442–446.
13. Sambol SP, Merrigan MM, Lyerly D, Gerding DN, Johnson S. Toxin gene analysis of a variant strain of *Clostridium difficile* that causes human clinical disease. *Infect Immun* 2000; 68: 5480–5487.
14. Barbut F, Lalande V, Burghoffer B, Thien HV, Grimprel E, Petit JC. Prevalence and genetic characterization of toxin A variant strains of *Clostridium difficile* among adults and children with diarrhoea in France. *J Clin Microbiol* 2002; 40: 2079–2083.
15. Brazier JS, Stubbs SL, Duerden BI. Prevalence of toxin A negative/B positive *Clostridium difficile* strains. *J Hosp Infect* 1999; 42: 248–249.
16. Kim H, Riley TV, Kim M *et al.* Increasing prevalence of toxin A-negative, toxin B-positive isolates of *Clostridium difficile* in Korea: impact on laboratory diagnosis. *J Clin Microbiol* 2008; 46: 1116–1117.
17. Johnson S, Sambol SP, Brazier JS *et al.* International typing study of toxin A-negative, toxin B-positive *Clostridium difficile* variants. *J Clin Microbiol* 2003; 41: 1543–1547.
18. Marsh JW, O'Leary MM, Shutt KA *et al.* Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in hospitals. *J Clin Microbiol* 2006; 44: 2558–2566.
19. van den Berg RJ, Claas EC, Oyib DH *et al.* Characterization of toxin A-negative, toxin B-positive *Clostridium difficile* isolates from outbreaks in different countries by amplified fragment length polymorphism and PCR ribotyping. *J Clin Microbiol* 2004; 42: 1035–1041.
20. van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. *J Clin Microbiol* 2007; 45: 1024–1028.
21. Fawley WN, Freeman J, Smith C *et al.* Use of highly discriminatory fingerprinting to analyse clusters of *Clostridium difficile* infection cases due to epidemic ribotype 027 strains. *J Clin Microbiol* 2008; 46: 954–960.
22. Brazier JS. Role of the laboratory in investigations of *Clostridium difficile* diarrhoea. *Clin Infect Dis* 1993; 16 (Suppl 4): S228–S233.
23. Kuijper EJ, Coignard B, Tull P. Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect* 2006; 12 (Suppl 6): 2–18.
24. Paltansing S, van den Berg RJ, Guseinova RA, Visser CE, van der Vorm ER, Kuijper EJ. Characteristics and incidence of *Clostridium difficile*-associated disease in the Netherlands, 2005. *Clin Microbiol Infect* 2007; 13: 1058–1064.
25. Bidet P, Lalande V, Salauze B *et al.* Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. *J Clin Microbiol* 2000; 38: 2484–2487.
26. Kato H, Kato N, Watanabe K *et al.* Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* 1998; 36: 2178–2182.
27. Kato H, Kato N, Katow S, Maegawa T, Nakamura S, Lyerly DM. Deletions in the repeating sequences of the toxin A gene of toxin A-negative, toxin B-positive *Clostridium difficile* strains. *FEMS Microbiol Lett* 1999; 175: 197–203.
28. Rupnik M, Avesani V, Janc M, Eichel-Streiber C, Delmee M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J Clin Microbiol* 1998; 36: 2240–2247.
29. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S–23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J Clin Microbiol* 1999; 37: 461–463.
30. Pituch H, vavn BA, van den BN *et al.* Clindamycin-resistant, toxin A-negative, toxin B-positive *Clostridium difficile* strains cause antibiotic-associated diarrhoea among children hospitalized in a haematology unit. *Clin Microbiol Infect* 2003; 9: 903–904.
31. Killgore G, Thompson A, Johnson S *et al.* Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. *J Clin Microbiol* 2008; 46: 431–437.
32. O'Neill GL, Beaman MH, Riley TV. Relapse versus re-infection with *Clostridium difficile*. *Epidemiol Infect* 1991; 107: 627–635.
33. Tang-Feldman Y, Mayo S, Silva JJ Jr, Cohen SH. Molecular analysis of *Clostridium difficile* strains isolated from 18 cases of recurrent *Clostridium difficile*-associated diarrhoea. *J Clin Microbiol* 2003; 41: 3413–3414.
34. van den Berg RJ, Ameen HA, Furusawa T, Claas EC, van der Vorm ER, Kuijper EJ. Coexistence of multiple PCR-ribotype strains of *Clostridium difficile* in faecal samples limits epidemiological studies. *J Med Microbiol* 2005; 54: 173–179.
35. Riggs MM, Sethi AK, Zabarsky TF, Eckstein EC, Jump RL, Donskey CJ. Asymptomatic carriers are a potential source for transmission of epidemic and non-epidemic *Clostridium difficile* strains among longterm care facility residents. *Clin Infect Dis* 2007; 45: 992–998.